Chapter 1

General introduction

Abstract

This chapter gives a general introduction to the fabrication of polar ordered peptide-based thin films and their intriguing features. The techniques to construct unidirectionally oriented α-helical polypeptide films are discussed and the Langmuir-Blodgett method to orient molecules at the air-water interface is described in more detail. The general structural properties and the synthesis of polypeptides as well as the progress in block copolypeptide synthesis are summarized. Recent developments in the construction and orientational control of Langmuir and Langmuir-Blodgett films of α-helical polypeptides are briefly reviewed. The aim and outline of this thesis is given at the end of this chapter.

1.1 Polar ordered peptide-based thin films

The α-helix, one of the fundamental secondary structures in proteins and peptides, shows a cumulative effect of individual dipoles of the peptide bonds in the main chain, resulting in a helix macrodipole.[1] Each peptide bond dipole contributes 3.46 Debye to the net dipole of the helix, with the direction oriented from the negatively charged C-terminus towards the positively charged N-terminus. However, the dipole moment is sensitive to polarization effects of the polar reaction field including solvent effects.[2-4] The α-helix dipole has been implicated in the structure stabilization and function of proteins and peptides.[5-8] It has also been demonstrated to affect the pK_a values in proteins,[9] to play a role in the anion binding of proteins and catalysis of enzymes[10] as well as in electron and proton transfer.[11-13]

The utilization of this bio-inspired functional unit in designing novel nanoscale active devices is one of the main challenges of this thesis.

Polymer brushes refer to an assembly of polymer chains densely end-grafted to a surface or interface.[14,15] Due to steric interactions, tethered chains are forced to stretch away from the surface. In this situation, the balance between repulsion and the elastic free energy of the chains leads to a higher energetic state than that of isolated chains. The chains in the brush may be attached to various interfaces by different mechanisms. For solid substrates, the chain end is grafted to the substrate via either chemical or physical bonds. The attachment of polymer chains to fluid interfaces may be achieved by different interactions of the chain end group, acting as an anchor, and the chain with the two media at either side of the interface, in which the end group prefers one medium and the chain prefers the other. Polymer brushes are an effective means to modify and tailor surface properties and have been shown to be useful for a broad range of physicochemical applications, including wetting, adhesion, colloid stabilization and biocompatibility.[15] They also provide a powerful tool to fabricate highly oriented thin films with control over surface chemistry and functionality for applications in actuation and sensing as well as nano- and biotechnology.[16,17]

When the α-helical chains are unidirectionally aligned via parallel chain fixation at a surface, the sum of non-cancelable helix macrodipoles results in a large net dipole. The orientation of helices at interfaces becomes one of the key factors to fabricate polar ordered bio-functional system. Oriented α-helical polypeptide monolayers have stimulated much interest because of their potential applications in chemical biology,[10] opto-electronics,[18-23] and biosensors.[24,25]

Studies have been made on the construction of polypeptide monolayers with oriented α-helices, mainly employing surface-grafting[10,11,18-47] and Langmuir-Blodgett (LB) techniques.[48-54] In the former, the polymer chains are chemically

attached to the surface of a solid substrate either by reaction between pre-formed polymers with modified end groups and a pretreated substrate containing reactive groups (grafting onto), or by polymerization of monomers initiated by initiator groups pre-attached to the substrate surface (grafting-from).[55] The helix orientation in grafted polypeptide films is governed by steric interactions between neighboring helices and hence is determined by the grafting density. To achieve a high grafting density and hence a high order of helix orientation, this method requires strict control over the reaction conditions (ultra-pure conditions), the density of reactive groups on the substrate surface and other factors such as steric hindrance and aggregations (in the grafting-on approach).[3,56,57]

The LB method organizes a single layer of molecules at an air-liquid or liquid-liquid interface before transfer onto a solid substrate. The method provides an efficient tool to construct thin films with molecular-level control over the structure.[58,59] The possibilities of structural order in two dimensions and layer-by-layer film deposition make the LB technique an excellent approach to model studies of membrane biophysics and two-dimensional (2-D) systems as well as for fabrication of functional supramolecular materials for optical, electronic and sensor applications.[60,61]

1.2 Langmuir-Blodgett (LB) technique

The LB method, named after Irving Langmuir and Katharine Blodgett,[62,63] provides a tool to fabricate films of controlled structure and thickness as well as possibilities for complex architectural design. Generally, a dilute solution of a surface-active low molecular weight or polymeric compound in a volatile organic solvent (immiscible with water) are spread on a liquid surface, usually water. After solvent evaporation, the molecules, which do not or barely dissolve in the adjoining phases, remain at the interface forming an insoluble monolayer or Langmuir monolayer. By compression of the Langmuir monolayer, a 2D molecular organization can be introduced.

The state of the monolayer at the interface can be monitored by measuring the surface pressure (π), defined as the difference between the interfacial tension with the presence of a monolayer (γ) and in the absence of the monolayer (γ_0): $\pi = \gamma_0 - \gamma$. The surface pressure-area (π-A) isotherm, π as a function of A at constant temperature, gives for a number of surface-active molecules information on molecular orientation at the interface and on molecular lateral interactions. The shape of the π-A isotherm for monolayers depends strongly on the molecular arrangement and the nature of materials.

Figure 1.1 shows a schematic example of a π-A isotherm of a Langmuir monolayer. Upon decreasing the available molecular area, the 2D monolayer goes through different phases: gas (G), liquid-expanded (LE), liquid-condensed (LC) and solid (S). The transition between two phases is indicated by a plateau, wherein they are in equilibrium. Extrapolation of the steepest part of the isotherm prior to collapse to zero pressure gives a minimum cross-sectional area of the molecule. However, in some cases, isotherms with features normally observed for monolayers may correspond to multilayers or incompletely spread films. To determine whether a monolayer is formed, complementary methods such as Brewster angle microscopy, fluorescence microscopy, X-ray reflectivity or diffraction are needed. Besides, temperature can also influence the π-A isotherm. Varying the temperature may change the monolayer phase.

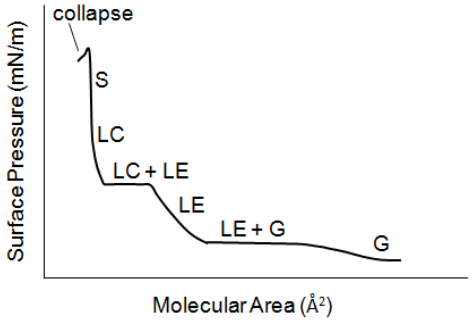


Figure 1.1. *Schematic example of a π-A isotherm.*

Monolayers of molecules can be transferred onto a solid substrate by the layer-by-layer deposition technique. Vertical deposition is the most common method and the transferred film is called a Langmuir-Blodgett (LB) film. Depending on the molecules spread at the air-water interface, deposition can result in different film architectures. The Y-type film, with alternating head-head and tail-tail orientations of the molecules, is formed when a monolayer is deposited during both down- and up-strokes and can be prepared either on hydrophilic or hydrophobic substrates. Other types of films, where tail-head interaction is more favorable, refer to deposition occurring only either during the upstroke (Z-type, hydrophilic substrate) or during the downstroke (X-type, hydrophobic substrate). Besides the vertical deposition, horizontal transfer of a Langmuir monolayer onto a solid substrate, known as the Langmuir-Schaefer (LS) deposition method,[64] is normally used for the deposition of rigid films. The advancements of the LB method inspired the pioneering work of Hans Kuhn and his group in the 1960s in constructing

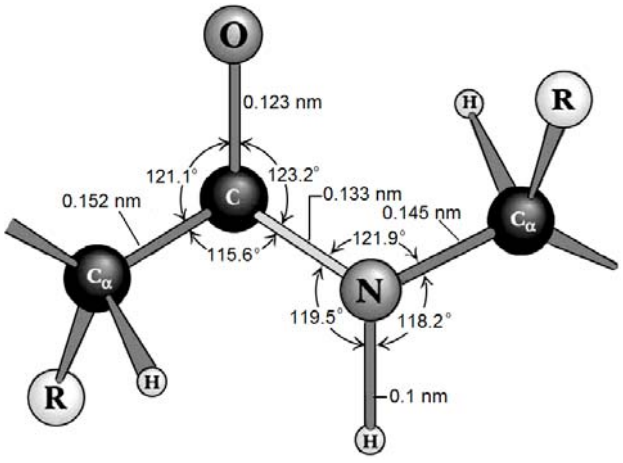
functional supramolecular assemblies.[65-67] The transferred films of functional molecules are also called by some authors Langmuir-Blodgett-Kuhn (LBK) films.

1.3 Polypeptides

Polypeptides and proteins are the building blocks of all living organisms and have a crucial biofunctional role.[68] Artificial polypeptides have received significant attention because of their potential for use in the construction of composites, supramolecular assemblies and biomedical applications.[3,69]

1.3.1 Polypeptide primary and secondary structures

The primary structure of a polypeptide or protein is the linear chain of α-amino acids, covalently linked through peptide bonds (Scheme 1.1). The length of the C-N bond, of 1.33 Å, is between that of a normal C-N bond (1.49 Å) and that of the C=N bond (1.27 Å). Because of the partial double bond character of the C-N bond, which restricts the rotation about the C-N bond, all four atoms of the peptide group (C, O, N and H) lie in the same plane.[70,71]



Scheme 1.1. *The peptide bond.*

The secondary structure is defined as a repetitive spatial arrangement stabilized by hydrogen bonding between the C=O and N-H groups of the peptide bonds. The most common secondary structures are the α-helix, β-pleated sheet (β-strand), β-turn and random coil (Scheme 1.2 and Scheme 1.3).

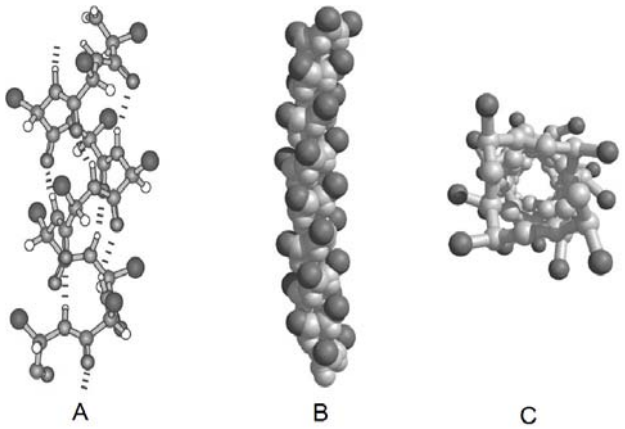
In the α-helix[72] the peptide units arrange in a regular right- or left-handed helical conformation, where the backbone C=O group of residue i hydrogen bonds with the N-H of residue i+4. There are 3.61 amino acid units per helix turn and the α-helix pitch per residue unit along the helix axis is 1.50 Å. Thus each turn contributes 5.41 Å to the total helix length.

Other rare helix secondary structures sometimes found at the ends of regular α-helices in proteins are the 3_{10}-helix and π-helix.[73] The 3_{10}-helix differs from the α-helix in that intramolecular hydrogen bonding occurs between residues i and i+3 instead of i and i+4 in α-helices. In the π-helix, the hydrogen bond is formed between residues i and i+5.

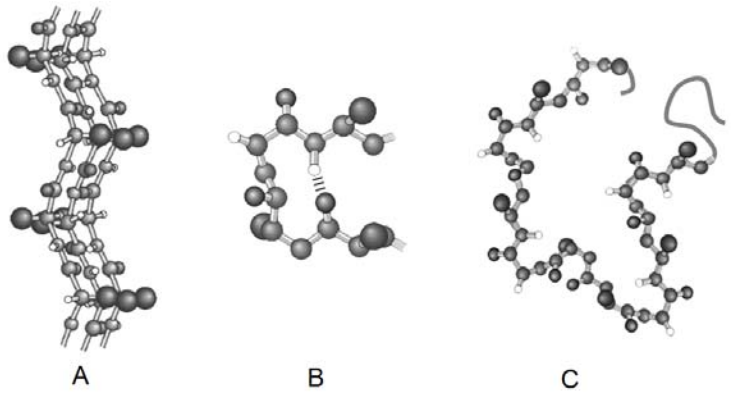
The β-sheet[74] is stabilized through intermolecular hydrogen bonds and van der Waals interactions between parallel or antiparallel aligned strands. The β-sheet has a zigzag structure giving a pleated appearance. Each residue has a translation length of 3.2 and 3.4 Å in antiparallel and parallel strands, respectively.

The β-turn is a local structure comprising of only four residues stabilized by a hydrogen bond between the C=O group of the first residue and the N-H group of the fourth residue, resulting in a reversal of the chain. β-Turns normally combine helices and sheets in proteins so that the peptide chains can fold back.

Polypeptide chains without a repetitive local structure are called random coils. This structure is formed when the hydrogen bonds stabilizing the secondary structures are disrupted. Selective solvents, pH and temperature are often used to manipulate the conversion between the α-helices or β-sheets and the random coil form of polypeptides.[4,75,76]



Scheme 1.2. *Schematic representation of the α-helix secondary structure: the ball-and-stick model (A), the rod-like structure (B) and the cross-sectional view (C).*

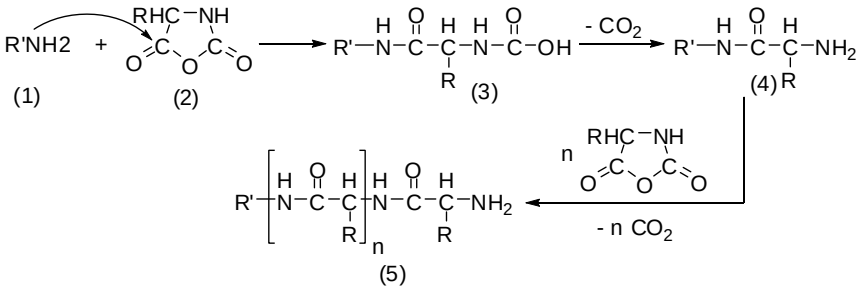


Scheme 1.3. *Schematic representation of the β-sheet (A), β-turn (B) and random coil (C) secondary structures.*

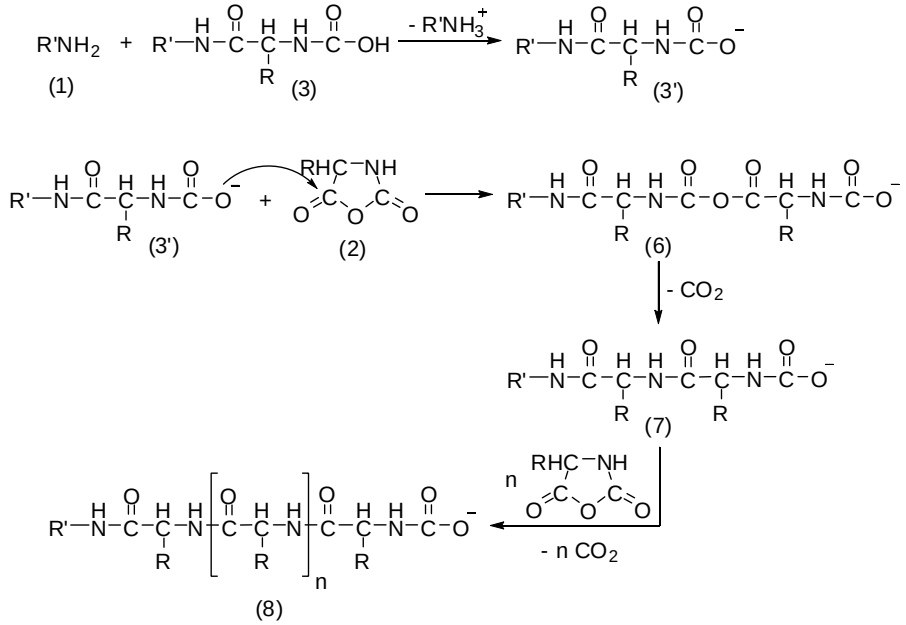
1.3.2 Mechanism of *N*-carboxyanhydride polymerization by basic initiators

The most widely used method to prepare synthetic polypeptides is the polymerization of *N*-carboxyanhydride (NCA), discovered by Leuchs[77] in 1906. The three classical mechanisms known for NCA polymerization by basic initiators (protic and aprotic amines, basic salts) are the "amine mechanism", the "carbamate mechanism" and the "activated monomer mechanism". Details about the synthesis, the polymerization mechanisms and kinetics can be found in reviews by Kricheldorf,[75,78] Bamford,[79] Sekiguchi[80] and Deming.[81]

The "amine mechanism"[82,83] is a nucleophilic ring opening polymerization process where the primary amine initiator is incorporated in the growing chain (Scheme 1.4). Because primary alkylamine initiators are somewhat more nucleophilic than the active amine chain end,[75,80] the polymerization has a "living character" if side reactions are absent. Nevertheless, in many cases when the primary amine is sufficiently strongly basic, the coexistence of the "carbamate mechanism" (Scheme 1.5),[84] which affects the polymerization kinetics, has been found.



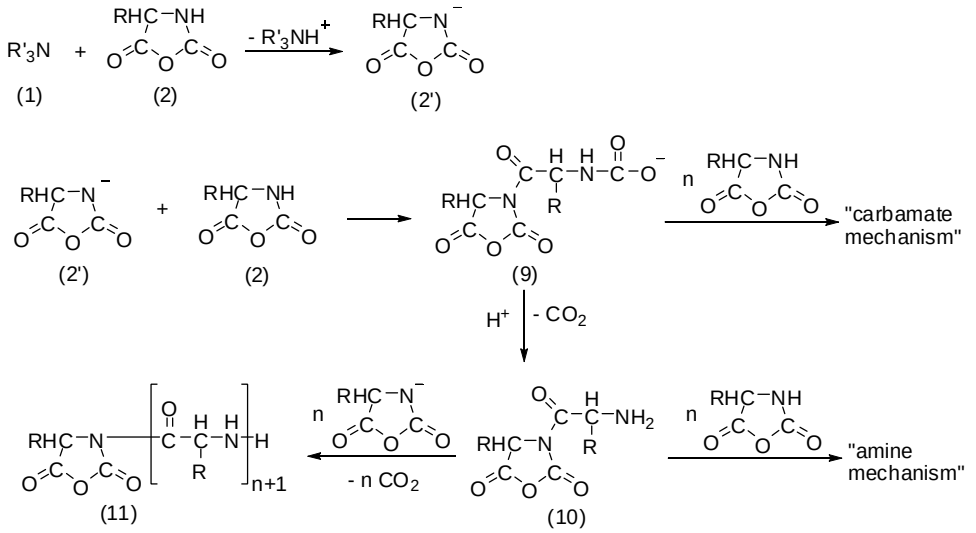
Scheme 1.4. *The "amine mechanism".*



Scheme 1.5. *The "carbamate mechanism".*

The "activated monomer mechanism",[85] usually known for the tertiary amine and alkali halide catalyzed polymerizations, occurs through the deprotonation of a NCA molecule by a strongly basic initiator to give an activated anionic monomer (Scheme 1.6). However, the polymerization does not take place exclusively through this mechanism, but proceeds via the various propagation reactions. The

high propagation-to-initiation rate constant ratio attributed to the "activated monomer mechanism" results in polymers with high molecular weights and large polydispersities.[75]



Scheme 1.6. *The "activated monomer mechanism".*

In the actual polymerization, the three mechanisms may coexist with different significance, depending on the nature of the NCA and initiator used. The propagation can switch back and forth between the mechanisms and a propagation reaction via one mechanism is a side reaction for the other.

In addition to the amine-initiated polymerization, NCA ring-opening polymerizations initiated by water and metal salts,[3,75] transition-metal amine complexes,[69] amine salts[86-88] and via genetic engineering[89] have been reported.

1.3.3 Block copolypeptide synthesis

During the past several years, different novel synthesis routes to block copolypeptides have been developed. A comprehensive review on recent developments in the synthesis of hybrid-peptide block copolymers has been given by Deming.[90] In general, the conventional and the most widely used method is the primary amine-initiated stepwise ring-opening polymerization of NCAs. Since the amine mechanism is a chain growth process and the reactive amine chain ends are preserved,[69,91] successive addition of different NCA monomers allows the

preparation of block copolymers. However, the drawback of the amine mechanism is that the polymerization is largely uncontrolled and hence yields polymers with polymerization degrees normally substantially different from the desired values with broad molecular weight distributions[92] and consisting of a large amount of dead chains (without amine end-groups).[93] Block copolypeptides prepared by this method have been reported to contain considerable homopolymer contaminants.[81,94]

To control NCA polymerization, Deming[95] developed transition-metal initiators, which activate the NCAs into covalent propagating species. Using a zerovalent nickel initiator in dimethylformamide, Deming was successful in the preparation of well-defined di- and tri-block copolypeptides of γ-benzyl-L-glutamate and ε-carbobenzyloxy-L-lysine.

Dimitrov and Schlaad[86,87] reported living polymerization of Z-L-lysine NCA with polystyrene-NH_2.HCl salt as a macroinitiator at increased temperatures (40-80 ^oC). The advantage of this method arises from reversible dissociation of the amine hydrochloride chain ends into the propagating free primary amine and $H^+(Cl^-)$ during the reaction, which suppresses side reactions via the "activated monomer mechanism".[75]

A patent by Breitenkamp and Sill[88] described the use of non-polymeric amine salts to initiate controlled ring-opening polymerizations of cyclic monomers. One of their examples is to use salts made from n-hexadecylamine and various acids such as hydrobromide, hydrochloride, acetic acid and formic acid as different initiators for sequential polymerizations of benzyl-glutamate NCA and butyl-tyrosine NCA in ***N***-methylpyrrolidone at 80 ^oC. However, though the polydispersities were small, the molecular weights of the products for each sequence were low compared to feeding ratios.

Another method leading to living polymerization of NCAs using primary amine initiators under high vacuum conditions was reported by the group of Hadjichristidis.[96,97] This technique allowed all reagents and the reaction environment to be of high purity and thereby eliminated side reactions. Block copolypeptides of various α-amino acid NCAs could be prepared with control over block lengths and small polydispersities.

A living polymerization of N_ε-trifluoroacetyl-L-lysine NCA using the n-hexylamine initiator at 0 ^oC was reported by Vayaboury et al.[98] By employing the non-aqueous capillary electrophoresis (NACE) technique, they could separate and quantify the amount of living chains (with amine end-groups) and dead chains. Interestingly, when conducting the reaction at such a low temperature, though the obtained product still had a double molecular weight distribution, only 1% of dead chains were formed. Although further study is required, this seems to be a promising route for preparation of block copolypeptides.

1.4 Langmuir and Langmuir-Blodgett (LB) films of α-helical polypeptides

Langmuir monolayers and LB films of synthetic polypeptides in the α-helical structure have been widely studied over the past decades.[99-123] The rigid-rod molecules lie flat on the water surface with a stable 2D nematic-like in-plane order of the α-helices. Using the LB technique, monolayers and multilayers of α-helical polypeptides can be transferred onto solid substrates. In-plane anisotropy can also be introduced by the LB vertical deposition method, wherein the transferred films exhibit a preferred orientation of polypeptide rods along the dipping direction.[109,124] The monolayer behavior of these rigid-rod polypeptides is strongly influenced by the side chain properties.[109,125]

The LB method has also been utilized for fabrication of polypeptide films with unidirectionally aligned molecules. Especially, the control of the molecular orientation at interfaces has been the subject of many studies on the biological function of supramolecular assemblies of peptides and proteins.[126,127]

Several polypeptide-based amphiphiles have been explored to achieve a perpendicular and unidirectional orientation of α-helices in polypeptide Langmuir monolayers. Kinoshita and his group[126] studied the orientation of various amphiphilic α-helical peptides at the hexane-water and air-water interfaces. The first reported systems were Langmuir monolayers of the amphiphilic diblock copolymers of poly(γ-methyl-L-glutamate) and polyethyleneglycol (PMG-PEG)[48] at the air-water interface and poly(γ-methyl-L-glutamate) bearing the hydrophilic β-cyclodextrin at the terminal (PMG-CyD) at the hexane-water interface.[49] A vertical helix orientation of these systems was suggested by the authors based on the consistency of the surface areas corresponding to the solid-state in the isotherms for the same type of polymers with different helix lengths. Nevertheless, the transferability and stability of helix orientation in the transferred films were not addressed.

The orientation of α-helical poly(L-leucine)-based amphiphiles in LB monolayers has been reported.[50,51] The helix orientation in these LB films was characterized by Fourier transform infrared reflection absorption spectroscopy (FT-IR/RAS). The helices of poly(L-leucine) having a trimethylammonium head group, though lying flat at the air-water interface, were found to be tilted at the hexane-water interface.[50] The LB monolayer of poly(L-leucine)-*b*-polyethyleneglycol diblock copolymers transferred from the air-water interface[51] was shown to have a helix orientation with a tilt at the interface. Yokoi et al.[52] controlled the helix orientation in the monolayer of a diblock copolypeptide consisting of poly(ε-benzyloxycarbonyl L-lysine) and poly(γ-methyl-L-glutamate-L-glutamic acid) at the air-water interface by manipulating the secondary structure of the hydrophilic

segment by varying the sub-phase pH. However, the transferred monolayers contained an amount of β-sheet and random coil structures. Thus the helix tilt angle, estimated to be smaller than 72°, was not calculated.

Niwa et al.[53] and Higashi et al.[54] reported on the orientation of amphiphiles consisting of poly(γ-benzyl-L-glutamate) (PBLG) using FT-IR/RAS. Niwa et al.[53] prepared an α-helix bundle structure by inducing helix association via ionic complexation of a quaternary ammonium-terminated PBLG (PBLG-N^+) with the sulfonate groups of bathophenanthroline disulfonate (PBS) and Fe-PBS. The helix orientation was manipulated by different complexation conditions. The monolayers of the PBLG-N^+ ion complexes, which were prepared in solution, exhibited a tilted helix orientation with a tilt angle of 41°. By performing the complexation between PBLG-N^+ and PBS at the water interface, the average helix tilt angle was decreased to 25°. A perpendicular helix orientation was claimed when PBLG-N^+/PBS was complexed with Fe-PBS dispersed in the water sub-phase.

Higashi et al.[54] reported the formation of a stable Langmuir monolayer of an amphiphilic diblock copolymer of PBLG and poly(L-glutamic acid) (PLGA) and transfer of the monolayer onto a hydrophobized substrate. The system was suitable for the enantiomeric capture of α-amino acids in aqueous solution by the PLGA segments. The tilt of helices in the transferred monolayers was found to vary with the transfer surface pressure and an average helix tilt angle of 33-45° was obtained at a range of pressures of 20-40 mN/m.

In conclusion, the LB method shows promise for the fabrication of biofunctional systems. The issue of interfacial molecular orientation in polypeptide assemblies is significant and deserves further research.

1.5 Aim and outline of the thesis

The aim of this study is to prepare α-helical amphiphilic diblock copolypeptides for the fabrication of double-brush LB films with a high polar order. The influence of polymerization conditions on the helicity of the diblock copolymers is investigated. The double-brush formation and helix orientation in Langmuir and LB monolayers are studied. Different physico-chemical phenomena concerning fabrication and manipulation of the diblock copolypeptide LB films are explored in order to fully understand and control the film structure and properties.

The synthesis of the diblock copolypeptides studied is described in Chapter 2. The effects of primary amine-initiated NCA polymerization conditions on the α-helix and β-sheet conformational contents of the diblock copolypeptides are explored. The reaction conditions are optimized to give purely α-helical diblock copolymers.

Chapter 3 studies the monolayer behavior of amphiphilic diblock copolypeptides at the air-water interface. An α-helical double-brush formation in LB monolayers is revealed.

Chapter 4 evaluates the surface potentials of α-helical amphiphilic diblock copolypeptides during monolayer compression at the air-water interface. The effective dipoles and their relation with the helix length in monolayers of unidirectionally aligned helices at the air-water interface are experimentally assessed for the first time.

Chapter 5 deals with the analysis of the helix tilt angles of the two blocks in Langmuir and LB monolayers of α-helical diblock copolypeptides. The formation of a smectic C-like phase in the monolayers is suggested. The chain length dependence of the helix orientation is demonstrated for the first time.

Chapter 6 reports on special effects in LB monolayers of α-helical amphiphilic diblock copolypeptides which are introduced by flow-induced orientation during the transfer process.

The effect of annealing on the structure and the helix orientation in LB monolayers of amphiphilic diblock copolypeptides is reported in Chapter 7.

Appendix 1 presents a preliminary investigation of the physical adsorption of *Candida antarctica* lipase B onto a double-brush LB monolayer of the amphiphilic diblock copolypeptide studied.

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